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Peri-implantitis-associated methanogens: a preliminary report

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Methanogens have already been described in periodontitis but not in peri-implantitis. Thirty peri-implantitis samples and 28 control samples were collected in 28 consenting peri-implantitis patients. PCR-sequencing of the 16S rRNA gene was used as a broad-spectrum screening method and results were further confirmed by real-time quantitative PCR targeting the *mcr*A genes. Results showed a methanogen community dominated by *Methanobrevibacter oralis* in 31/58 (51%) samples including 16/28 (57%) control samples and 15/30 (50%) peri-implantitis samples. *Methanobrevibacter massiliense* was detected in 5/58 (8.6%) samples including 3/28 (1%) control samples and 2/30 (6.7%) peri-implantitis samples. The prevalence of *M. oralis* or *M. massiliense* did not significantly differ in peri-implantitis and control samples (exact Fisher test, P = 0.61 and P = 0.67, respectively). Further ponderation of the methanogen load by the real-time quantitative PCR for actin human gene again indicated non-significant difference (Wilcoxon-Mann-Whitney test, P = 0.48 and P = 0.40, respectively). These data show that the prevalence of methanogens does not differ in peri-implantitis lesions and healthy sites, when individuals are their own control. These data do not allow assigning a specific pathogenic role to methanogens in peri-implantitis; methanogens rather are part of the commensal and normal flora of the oral cavity.

Peri-implantitis is an inflammatory disease characterized by the destruction of soft and hard tissues and the formation of pockets around osteo-integrated dental implants[1]. Contrasting with metagenomics studies which failed to detect any methanogen in peri-implantitis lesions[2,3], methanogen-targeting molecular investigations detected DNA sequences specific for the methanogens *Methanobrevibacter oralis*, *Methanobrevibacter smithii*, *Methanomassiliicoccus luminyensis*, "*Methanobacterium congolense/curvum*" and *Methanobrevibacter massiliensis* in parodontitis pockets[4,5]. *M. oralis*, an oral cavity inhabitant[6] has been more frequently found in parodontitis pockets[7] in association with anaerobes[8] and its role was analyzed as moderate[7]. Yet, studies relying on the simple, un-quantified molecular detection of sequences leaved unsatisfied the relative role of these methanogens in the pathogenesis of peri-implantitis[9]. We hypothetized that the relative load of methanogens among the total flora in peri-implantitis lesions, would give an indication on the relative role of methanogens in the pathogenesis of peri-implantitis as previously showed for periodontitis[10].

Patients and Methods

Twenty-eight patients diagnosed with peri-implantitis were prospectively included in this study from January 2016 to June 2016. This study was approved by the clinical research ethics committee of the IFR 48 of the University of Aix-Marseille approved (protocol No. 2016–011). All methods were performed in accordance with the relevant guidelines and regulations. The study protocol was explained to each patient and all patients signed an information document and an informed consent document. All patients were interviewed in order to collect their medical and dental history including the date of implantation. An intra-oral examination was performed to measure bleeding on probing (BOP), probing depth (PD), the surfaces with presence of plaque (%), the presence of pus, the number of implants affected by the peri-implantitis, the presence of mobility and the vertical resorption of the supporting bone. In addition, a radiographic status and a 3D radiography were realized. Patient was then diagnosed with periimplantitis when the following criteria were met: (1) vertical resorption of the supporting bone ≥2 mm or (2) probing depth ≥5 mm or (3) bleeding or (4) pus or (5) motility of the implant according to the literature[11]. Subgingival dental plaque samples were collected from all peri-implantitis pockets of each individual with sterile Gracey curettes 1/2 (Hu-Friedy, Rotterdam, Netherlands) and placed into

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tubes containing 1 mL of the SAB anoxic transport medium for methanogens composed of 0.1 g/L; MgCl, 0.2 g/L; KH_2PO_4, 0.2 g/L; KCl, 0.1 g/L; $CaCl_2$, 3 g/L; NaCl, 1.15 g/L; Na_2HPO_4, 1 g/L; ascorbic acid, 1 g/L; uric acid, 1 g/L; glutathione, pH 7.5 with 0.3/0.6 g/L; KOH[12]. Negative control subgingival dental plaque samples were collected from healthy gingival sites with another curette of Gracey sterile and places in the same medium. Each sample collected was suspended in 1 mL transport medium. After homogenization, a 250-μL aliquot was shaken with 0.3 g of acid-washed beads (≤106 mm; Sigma, Saint-Quentin-Fallavier, France) in a FastPrep-24 5 G™ instrument (MP Biomedical Europe, Illkirch, France) at a speed of 6.5 m/s (full speed) for 90 s. The supernatant was incubated overnight at 56 °C with 180 μL lysis buffer and 25 μL proteinase K (20 mg/mL) in the Qiagen EZ1 DNA tissue kit (Qiagen, Courtaboeuf, France) and total DNA was then extracted using a kit NucleoSpin® NucleoSpin® Tissue kit (MACHEREY- NAGEL, Duren Germany; REF: 74095250). Extraction DNA realize after adding 200 μL B3 buffer, 10 μL synthetic plasmid and incubated for 60 minutes at 70 °C. DNA was then washed with 200 μL BW buffer and 600 μL B5 buffer (MACHEREY-NAGEL). After centrifugation for 1600 g, the DNA pellet was eluted in 100 μL of EB buffer (heated to 70 °C) (MACHEREY-NAGEL). As for PCR amplifications, negative controls consisting of sterile DNA-free water were introduced in all the manipulations. We have performed an amplification of the external synthetic plasmid to check for inhibitor absence in PCR. In the first broad-spectrum screening step, methanogen DNA was tentatively detected using PCR-sequencing of the methyl-coenzyme M reductase (*mcr*A) gene (primers mcrALuF 5′-GGTGGTGTMGGATTCACACARTAYGCWACAGC-3′ and mcrALuR 5′-TTCATTGCRTAGTTWGGRTAGTT-3′) and the 16S rRNA gene (primers: 16SrDNA SDArch0333aS15-F 5′-TCCAGGCCCTACGGG-3′ and 16SrDNA SDArch0958aA19-R 5′-YCCGGCGTTGAMTCCAATT-3′) as previously described[13]. The PCR program was 94 °C for 30 s, 58 °C for 45 s and 72 °C for 30 s followed by 35 cycles of 10 min at 72 °C for amplifying the16S rRNA gene; and 40 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s followed by extension of 10 min at 72 °C for amplifying the *mcr*A gene. PCR product sequences were analyzed with the ChromasPro program, version 1.7: sequence similarity values were determined by BLAST program in the online analysis platform from NCBI (blast.ncbi.nlm.nih.gov). In the second step, specific quantitative amplification of the *M. oralis* strain JMR0[14] DNA was achieved using a specific real-time PCR assay targeting *M. oralis* heat-shock protein *cnp*60 gene as previously described[3]. Briefly, *M. oralis cnp*602P probe (6-carboxyfluorescein (FAM)-5′AGCAGTGCACCTGCTGATATGGAAGG-3′) (Eurogentec, Seraing, Belgium), *M. oralis-cnp*602F primer (5′-GCTGGTGTAATCGAACCTAAACG-3′) and *M. oralis-cnp*602R primer (5′-CACCCATACCCGGATCCATA-3′) (Eurogentec) were incorporated into a PCR program comprising of 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 35 s and 45 °C for 30 s. The real-time PCR was regarded as positive for any cycle threshold (Ct) value ≤40. A calibration curve was established by measuring the Ct values for serial ten-fold dilutions of *M. oralis* (10E + 01 to 10E + 09). Stock inoculum of 10^9 *M. oralis* organisms (as determined by optic density measurement = 0.4) was serially diluted in sterile PBS. Then, each dilution was run in triplicate in order to determine the calibrating curve. Further, specific quantitative amplification of the *M. massiliense* DNA targeted the *M. massiliense mcr*A gene. Primer sequences were determined by using the software primer3 after alignment of the *mcr*A gene sequence from *M. massiliense*, *M. oralis*, *M. smithii*, *M. arboriphilus*, *Methanobrevibacter ruminantium*, *Methanobrevibacter wolinii*, *Methanobrevibacter cuticularis*, *Methanobrevibacter filiformis*, *Methanobrevibacter millerae* and *Methanobrevibacter olleyae*. This yielded a *M. massiliense mcr*A probe (6-carboxyfluorescein (FAM) 5′-TGG-CTG-TTC-CAC-TGC-ATT-CGCT -3′) and the primer pair *M. massiliensis-mcrA* F (5′-ACTCACTTTGGCGGATCTCA-3′) and *M. massiliensis-mcr*A R (5′-GTACATGGACAAGTACCATGC-3′). The PCR program was 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 35 s and 45 °C for 30 s. A calibration curve was established by measuring the Ct values of ten-fold serial dilutions of *M. massiliensis* (10E + 01 to 10E + 05). Furthermore, real-time PCR targeting the human actin gene was used as an internal calibrator using primer pair Actin F: 5′CATGCCATCCTGCGTCTGGA3′ and ActinR: 5′CCGTGGCCATCTCTTGCTCG3′ (Eurogentec) and the Actin P6FAM-probe: 5′CGGGAAATCGTGCGTGACATTAAG3′ (Eurogentec). The PCR program was 50 °C for 2 min, 95 °C for 3 min, followed by 45 cycles of 95 °C for 15 s, 60 °C for 30 s and 45 °C for 30 s. A calibration curve was established by measuring the Ct values of serial ten-fold dilutions of HL-60 cells (human leukemia cells) (4.10E + 00 to 4.10E + 05). Finally, the load ratio of *M. oralis*/human cell and *M. massiliense*/human cells was calculated by dividing the extrapolated number of methanogens in the sample by the extrapolated number of cells using actin quantification as a proxy.

Differences in the prevalence of methanogens in the peri-implantitis sample group and the control sample group were analyses for statistical significance by using the Fisher exact test for discrete values and the Wilcoxon-Mann-Whitney test for ratios; a $P \leq 0.05$ was used to infer significance.

Availability of data and materials. The datasets generated and/or analyzed during the current study are available within the manuscript.

Results

Eight men and twenty women aged between 35 and 86 years diagnosed with peri-implantitis were prospectively included in the study. One control sample and one peri-implantitis sample were collected in each patient, except for patient no. 24 in whom three peri-implantitis samples were collected; giving a total of 28 control samples and 30 peri-implantitis samples. A total of 10 individuals had generalized bleeding while 18 had localized bleeding. The pocket depths ranged from 4 to 12 mm (x^-: 7.14, σ: 1.99). The number of implants affected by peri-implantitis ranged from 1 to 7 (x^-: 2.64, σ: 1.80). In four implants diagnosed with peri-implantitis, pus was observed. The vertical resorption of the supporting bone (% of implant length) ranged from 29% to 100% (100% in only one case) (x^-: 54.64, σ: 17.69) (Table 1). In all PCR experiments, negative controls remained negative and the positive amplification of the external synthetic plasmid control demonstrated the absence of PCR inhibitors. Using methanogen-specific PCR amplification, 31/58 samples (53.4%) were positive for *M. oralis* with

Patient	Sample	Sexe	BOP	PD (mm)	Surfaces with presence of plaque (%)	Presence of pus	Number of implants affected by periimplantitis	Presence of mobility	Vertical resorption of the supporting bone (% of implant lenght)
1	P1	f	Generalized	8	50	No	4	No	48
2	P2	f	Generalized	7	100	Yes	2	No	70
3	P3	f	Generalized	7	25	No	1	No	51
4	P4	m	Localized	4	100	No	2	No	37
5	P5	m	Localized	6	100	No	7	No	46
6	P6	f	Localized	4	50	No	2	Yes	29
7	P7	f	Generalized	7	50	No	3	Yes	69
8	P8	f	Localized	8	25	Yes	1	No	51
9	P9	f	Localized	5	25	No	2	No	64
10	P10	m	Localized	4	25	No	1	No	46
11	P11	f	Localized	7	100	Yes	2	No	71
12	P12	f	Localized	12	25	No	1	Yes	100
13	P13	f	Localized	10	50	No	5	No	60
14	P14	f	Generalized	9	100	No	2	Yes	74
15	P15	f	Localized	6	100	No	1	No	52
16	P16	m	Generalized	5	100	Yes	6	No	35
17	P17	f	Localized	7	25	Yes	7	No	67
18	P18	f	Localized	8	100	Yes	1	No	60
19	P19	m	Generalized	7	100	Yes	4	No	57
20	P20	f	Localized	10	100	Yes	2	No	81
21	P21	f	Localized	6	50	No	1	No	50
22	P22	m	Localized	6	100	Yes	1	No	35
23	P23	f	Localized	9	100	No	2	No	40
24	P24, P25, P26	f	Generalized	10	100	No	4	No	85
25	P27	m	Localized	6	100	No	2	No	36
26	P28	m	Generalized	7	100	Yes	2	No	35
27	P29	f	Generalized	9	50	No	4	No	46
28	P30	f	Localized	6	50	No	2	No	35

Table 1. Clinical data collected in patients with periimplantitis. BOP, bleeding on probing; PD, probing depth.

	Peri-implantitis			Healthy control		
Samples	*M. massiliense* Load	Human cells load	*M. massiliense*/ Actin ratio	*M. massiliense* load	Human cells Load	*M. massiliense*/ Actin ratio
4	2,06E + 02	7,31E + 02	2,82E − 01	1,08E + 02	8,87E + 02	1,22E − 01
8	7,02E + 03	6,31E + 01	1,11E + 02	5,08E + 03	2,21E + 02	2,30E + 01
18	N/A	6,89E + 05	N/A	3,33E + 03	1,80E + 04	1,85E − 01

Table 2. Ratio *Methanobrevibacter massiliense* load/human cells load.

100% gene sequence similarity with the reference 16S rRNA and *mcr*A gene sequences; comprising 16/30 (53%) peri-implantitis positive samples and 15/28 (53.6%) control samples. Further, 5/58 (8.6%) samples were positive for *M. massiliense* with 100% 16S rRNA and *mcr*A gene sequence similarity with reference sequences[9] including 2/30 peri-implantitis samples and 3/28 control samples (Table 2). Altogether, methanogens were detected in 36/58 (62%) samples including 17/30 (57%) peri-implantitis samples and 19/28 (67.8%) control samples. Further *M. oralis* and *M. massiliense*-targeted PCR amplifications confirmed all the negative and positive results obtained by using methanogen-specific PCR amplifications. There was no statistically significant difference in the prevalence of methanogens in the peri-implantitis and control samples (*M. oralis*, $P = 0.61$; *M. massilense*, $P = 0.67$; all methanogens, $P = 0.45$).

Further detection of actin by qPCR extrapolated into 1.15E + 01–9.29E + 05 cells/sample (x^{-}: 7.71E + 04, σ: 1.89E + 05) (Tables 2 and 3). Actin measurement based deriving the load of host cells which calculated to be (x^{-}: 1.04E + 05, σ: 2,33E + 05) in the peri-implantitis samples and (x^{-}: 4.86E + 04, σ: 1,23E + 05) in the control specimen for positive sample. Methanogen DNA was detected in 19/28 (67%) control samples with an average number of cells of (x^{-}: 1.42E + 04, σ: 4.94E + 04) and in 17/30 (60%) in the peri-implantitis sample with an average number of cells of (x^{-}: 8.03E + 05, σ: 2,53E + 06). The mean load ratio of *M. oralis* or *M. massiliense* in the peri-implantitis samples was not significantly different than in the control samples ($P = 0.48$ and $P = 0.4$, respectively).

Samples	Peri-implantitis			Samples	Healthy control		
	M. oralis load	Human cells Load	*M. oralis*/Actin ratio		*M. oralis* load	Human cells Load	*M. oralis*/Actin ratio
1	N/A	2,00E+01		1	5,74E+02	1,66E+02	3,46E+00
2	6,53E+04	6,10E+01	1,07E+03	2	1,33E+05	1,96E+01	6,79E+03
3	N/A	2,74E+00		3	N/A	9,11E+01	
4	N/A	7,31E+02		4	N/A	8,87E+02	
5	1,52E+08	1,51E+01	1,01E+07	5	3,92E+02	1,17E+02	3,35E+00
6	4,22E+03	7,07E+01	5,97E+01	6	N/A	2,56E+01	
7	N/A	2,26E+01		7	5,04E+04	1,15E+01	4,38E+03
8	N/A	6,31E+01		8	N/A	2,21E+02	
9	1,86E+04	1,81E+01	1,03E+03	9	5,98E+05	1,37E+01	4,36E+04
10	2,69E+02	3,80E+01	7,08E+00	10	1,47E+02	2,89E+01	5,09E+00
11	2,08E+05	2,51E+01	8,29E+03	11	1,50E+03	2,87E+01	5,23E+01
12	N/A	2,21E+01		12	7,09E+02	1,51E+01	4,70E+01
13	2,42E+02	3,13E+02	7,73E-01	13	N/A	7,86E+01	
14	N/A	1,01E+04		14	N/A	1,62E+01	
15	N/A	1,86E+02		15	N/A	1,63E+04	
16	N/A	6,19E+04		16	N/A	2,14E+02	
17	2,27E+09	1,74E+04	1,30E+05	17	3,83E+03	3,05E+03	1,26E+00
18	1,06E+02	6,89E+05	1,54E-04	18	N/A	1,80E+04	
19	N/A	5,91E+05		19	1,19E+03	1,12E+05	1,06E-02
20	1,23E+09	3,61E+02	3,41E+06	20	6,58E+05	3,31E+05	1,99E+00
21	6,58E+05	6,89E+02	9,55E+02	21	2,43E+05	7,61E+02	3,19E+02
22	N/A	7,41E+02		22	N/A	3,05E+03	
23	N/A	4,04E+04		23	7,15E+06	4,55E+04	1,57E+02
24	N/A	2,04E+05		24	N/A	3,40E+05	
25	4,48E+05	2,71E+05	1,65E+00	25	7,99E+04	1,17E+03	6,83E+01
26	N/A	9,49E+05		26	N/A	6,14E+02	
27	4,40E+05	2,04E+03	2,16E+02	27	6,23E+07	2,91E+02	2,14E+05
28	N/A	2,85E+03		28	5,74E+02	4,87E+05	1,18E-03
29	6,99E+07	2,38E+05	2,94E+02				
30	1,46E+02	3,37E+04	4,33E−03				

Table 3. Ratio *Methanobrevibacter oralis* load/human cells load. N/A, not available.

Discussion

In this study, we detected the same prevalence of *M. oralis* and *M. massiliensis's* DNA in control samples as in the peri-implantitis samples. Both types of samples originated from the same patient, which means that every patient was also his own control. Using this strict methodology and appropriate negative controls which remained negative in all experiments, we observed that the load of both methanogens was not significantly different in the peri-implantitis samples than in the control samples. These results agree with a previous study detecting *M. oralis* in 10 patients with aggressive periodontitis and in 10 periodontally healthy individuals[6]. In a more recent study, *M. oralis* respresented 82% of cloned methanogen DNA in patients and 70.1% in healthy individuals[6]. These results however differ from those previously reported using 16S rRNA gene clone library analysis, which failed to recover any methanogen sequences in peri-implantitis samples[3]. We proposed that differences in the methodology partly account for differences in the reported results. Indeed, detecting *M. oralis* in peri-implantitis lesions is not surprising as *M. oralis* is the main methanogen detected in the oral cavity with a prevalence higher than 40%[15]. Here, *M. oralis* was detected in 53.4% (31/58) of samples. In addition, we also detected *M. massiliense* previously reported in the oral cavity with prevalence <20%[8,9].

However, we propose that the major difference between previous reports and our present report relies on the choice of the negative controls, i.e. using individuals as their own control as we invented here. Our choice to sample infected (diseased) and non-infected control site within the same person was based on the fact that "the controls are controls only if they are healthy"[11]. However, this choice exposes to the possibility of overmatching bias which occurs when controls become more like the cases about exposure than the general population[11]. Indeed, sampled sites are in the same environment with similar exposure by the same microbiota as mentioned in the situation of overmatching bias[16]. Potential overmatching bias has to be taken into consideration to interpret the data here reported. With the reserve of a potential overmatching bias, our analyses showed an absence of any difference in the prevalence and in the load of methanogens in peri-implantitis samples and control samples collected in the very same person. Furthermore; a study has shown that the environment in the sulcus depths of peri-implantitis is well adapted to the growth of obligate anaerobic bacteria which provides a specific signature during this infection, the microorganisms of the oral microbiota within the same oral cavity are not exposed to the same environment; oral microbiota and disease sites versus oral microbiota and healthy sites[8]. We argue that

Archaeal genus/species	Detection Methods	Infections	Prevalence in patients	Prevalence in healthy subjects	References
Methanobrevibacter oralis	Culture, PCR, FISH	Periodontitis	nt	04/10	19
			18/50	00/8	20
			11/49	00/17	21
			44/102	00/65	22
			12/25	03/25	9
			02/30	nt	23
			03/12	nt	24
	PCR Culture	Brain Abscess	+	nt	17
Methanobrevibacter smithii	Culture, PCR	Periodontitis	+	nt	25
			09/20	nt	26
			+	nt	27
		Chronic paravertebral muscle abscess	+	nt	18
Methanosphaera stadtmanea	Culture	Periodontitis	+	nt	25
Methanosarcina spp./M. mazeii	Culture, PCR	Periodontitis	01/17	nt	28
			+	+	6
Thermoplasmata spp./Thermoplasmatales	PCR	Periodontitis	+	−	15
			03/30	nt	23
Methanobacterium curvum/congolense	PCR	Periodontitis	04/25	02/25	9
			+	+	6

Table 4. List of studies reporting the tentative detection of methanogens in diseased and healthy subjects. +Detection; −No detection; nt, not tested.

these controls are of better value than control samples collected in another individual, as previously reported in studies of peri-implantitis and in studies of periodontitis[6]. The possibility that methanogens from diseased tissues did contaminate healthy tissues cannot be ruled out in our study. This would obviously temperate the conclusions here reported.

Methanogens have been previously detected in another, closely related situation of periodontitis; and that their load was significantly associated with the severity of the periodontal lesions. The results here reported therefore mirror and extend previous data to a yet un-explored situation of peri-implantitis. Indeed, the microbial flora recovered from peri-implantitis lesions differs from that of periodontitis. The genus *Olsenella*, *Sphingomonas*, *Peptostreptococcus* and unclassified *Neisseriaceae* are more abundant in peri-implantites whereas the genus *Desulfmicrobium* is lower. The methanogens, although unable to detect species, are more important in peri-implantitis than periodontitis but without significant difference[13]. In these situations, it has been postulated that the particular metabolism of methanogens, which reduces CO_2 with H_2 as the electron donor for methanogenesis, favors the growth of pathogenic bacteria in anaerobic gingival pockets[6]. Also, methanogens including *M. oralis* and *M. smithii* have been detected and cultured in anaerobe abscesses, including several cases of life-threatening brain abscess[17] and in one case of muscular abscess[18]. These clinical observations along with experimental data in *M. oralis* –challenged mice[17] undoubtedly indicate that methanogens are opportunistic pathogens (Table 4).

Here, we observed that the methanogen load but not the sole detection of methanogens, was not significantly associated with peri-implantitis. This observation differs from that drawn from a previous comparative study of peri-implantitis sites, healthy implant sites and healthy sites[9]. In latter study, authors found a significant difference in the prevalence of methanogens in peri-implantitis sites (48%) and in healthy implants (16%) and natural teeth (8%). Nevertheless, we argue that detecting the presence of methanogens alone is not sufficient to diagnose peri-implantitis, which is probably due to their universal presence in the oral cavity, producing an unavoidable contamination of samples. Only a strict quantification of those methanogens would allow us to overcome this issue. Indeed, we previously made exactly the same observation as for periodontitis[7].

Further studies are warranted to ensure that the observations here reported are not restricted to our center. Indeed, other populations with other geographical and nutritional backgrounds may exhibit different repertoires of methanogens. Cumulative studies will refine our knowledge of the potential role of methanogen in mixed infection with anaerobes in the pathology of peri-implantitis, guiding therapeutic proposals. In any case, peri-implantitis is a complex pathology which definite diagnosis does not rely on the detection of any single microorganism.

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E.T., M.D., G.A. conceived the study and designed the methodology. S.B., A.M., D.T., P.T., G.S., G.B. performed the experiments and E.T., G.A. and M.D. analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Additional Information
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